The power of the DIG System

The DIG System for nucleic acid analysis can be used for single-copy gene detection on human genomic Southern Blots, the detection of unique mRNA species on Northern Blots, colony and plaque screening, Dot Blots and *in situ* hybridization. Examples and protocols for these applications (except for *in situ* hybridizations) can be found throughout The DIG User's Guide. For a comprehensive treatment of nonradioactive *in situ* hybridization, ask for a free copy of Boehringer Mannheim's "Nonradioactive *in situ* Hybridization Manual."

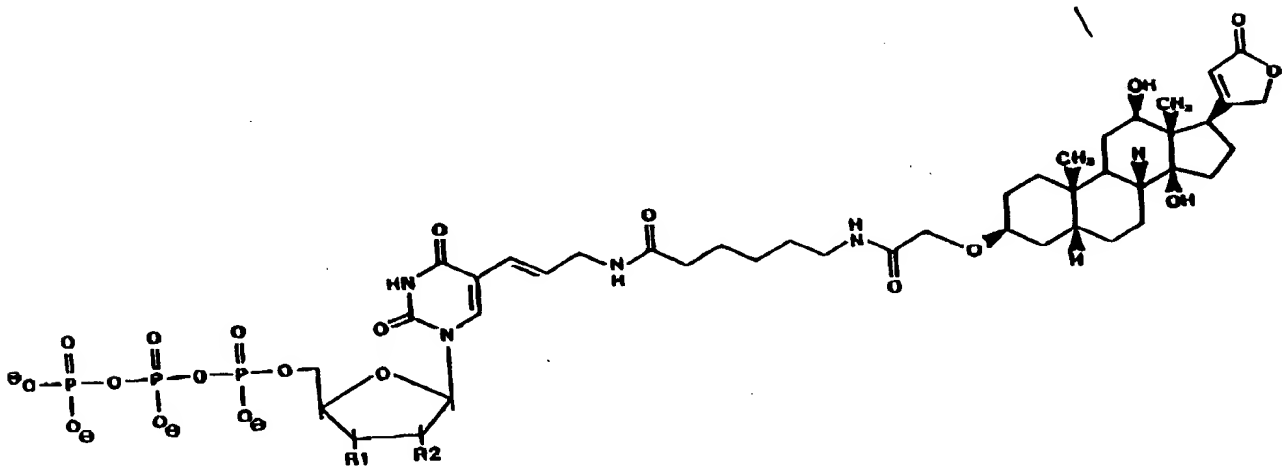
Use of the User's Guide

This manual describes all the above labeling methods and all the alkaline phosphatase-based detection assays. All the labeling and detection methods are presented in one manual so that users of the DIG System have a convenient reference to base their experiments on. For an overview of the DIG hybridization experiment, see the scheme presented in Figure 2.

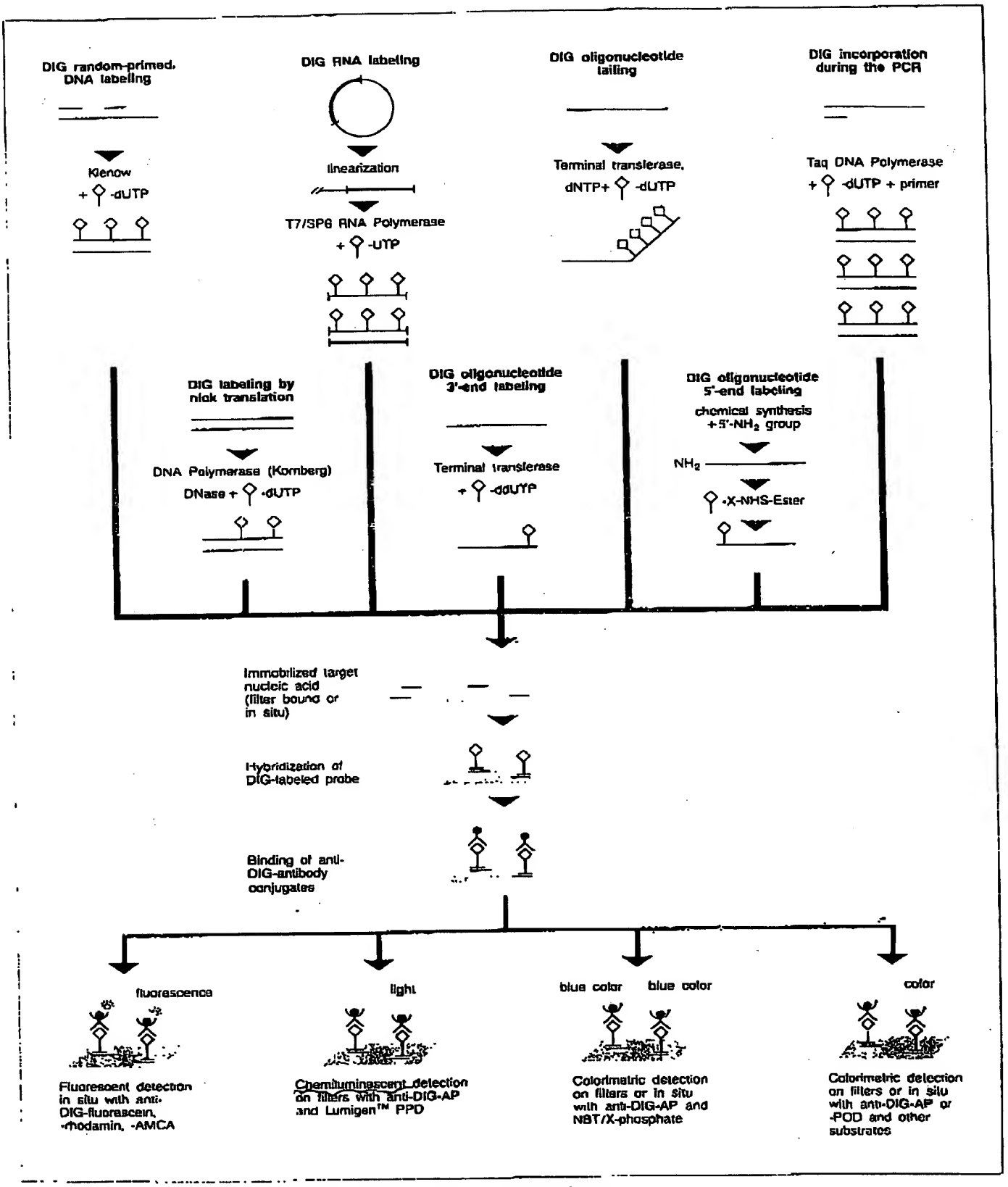
Principle of Nucleic Acid Labeling and Detection with the DIG System

The DIG System uses digoxigenin, a steroid hapten, to label DNA, RNA or oligonucleotides for hybridization and subsequent detection. DNA probes are labeled with DIG-11-dUTP via random primed labeling, nick translation, DNA synthesis or Taq DNA polymerase. Oligonucleotide probes are 3'-end labeled with DIG-11-ddUTP, tailed with DIG-11-dUTP by Terminal transferase, or labeled at the 5'-end with digoxigenin-NHS ester. RNA can be labeled with DIG-11-UTP by SP6, T3, or T7 RNA polymerase in an *in vitro* transcription reaction. The DIG-labeled probes are hybridized to a membrane-bound nucleic acid on a Northern blot, Southern blot, Dot/Slot blot or colony/plaque lift. These hybridized probes are immuno-detected with an alkaline phosphatase-conjugated anti-digoxigenin antibody and then visualized with the chemiluminescent substrate CSPD®, with the colorimetric substrates NBT and X-phosphate or "Fast Dyes" (Multicolor Detection Set).

Please note:
The chemiluminescent AP-substrates AMPPD®, Lumigen PPD™ and Lumi-Phos 530 which were previously supplied by Boehringer Mannheim have recently been replaced by CSPD®. Compared to other 1,2-dioxetanes (e.g. AMPPD®) CSPD™ provides higher sensitivity due to significantly reduced background. Its fast kinetics of light emission permit maximum signal to be attained in less than 10 min exposure time. The reaction conditions are the same as for AMPPD® or Lumigen PPD™ and are described in detail on page 47 (Detection) of this manual.
Since the experimental results provided by our customers were obtained before the switch to CSPD®, most of the experiments were done with Lumigen PPD™. According to our own experiences the results would look the same (band intensity might be slightly increased) if CSPD® was used.



▲ **Figure 3:** Digoxigenin-UTP (R1 = OH, R2 = OH)/Digoxigenin-dUTP (R1 = OH, R2 = H)/Digoxigenin-ddUTP (R1 = H, R2 = H)



▲ **Figure 4:** The labeling and detection alternatives offered by the DIG System

Hybridization

General Considerations for Hybridization

Membrane filters

For best results, Boehringer Mannheim's nylon membranes, positively charged (Cat. Nos. 1209299, 1209272, 1417240) should be used for the transfer. This membrane has an optimal charge density, allowing the nucleic acid to bind tightly without producing high background. Furthermore our nylon membrane is specifically tested with the DIG system to ensure optimal sensitivity and background characteristics.

Note: All tested nitrocellulose membranes work well with the DIG system. However, nylon membranes must be used for chemiluminescent detection or if stripping or reprobing is to be performed. Nitrocellulose membranes will not work in these applications.

Optimization of the probe concentration - the "mock" hybridization

To prevent background problems, we recommend the probe concentration to be optimized before hybridization. The mock hybridization is especially important when performing chemiluminescent detection.

The mock hybridization is carried out by incubating small membrane pieces (without DNA) with different probe concentrations in the hybridization solution and afterwards detecting according to standard procedure.

For example:

Probe type	Concentration in the hybridization solution		
DNA/RNA probes	1 µl*/ml	3 µl*/ml	5 µl*/ml
End labeled oligonucleotide	1 pmol/ml	3 pmol/ml	10 pmol/mol
Tailed oligonucleotide	0,1 pmol/ml	0,5 pmol/ml	2 pmol/ml

*from the labeling reaction

The highest probe concentration which gives an acceptable background should be used for the hybridization experiment.

Prehybridization/hybridization buffers

Many different prehybridization and hybridization buffers can be used with the DIG system. We can recommend 3 options:

- **Standard buffer**
  5 x SSC
  0.1% (w/v) N-lauroylsarcosine
  0.02% (w/v) SDS
  1% Blocking Reagent
- **Standard buffer + 50% formamide**
  50% Formamide, deionized
  5 x SSC
  0.1% (w/v) N-lauroylsarcosine
  0.02% (w/v) SDS
  2% Blocking Reagent
- **"High SDS buffer"**
  7% SDS
  50% formamide, deionized
  5 x SSC
  2% Blocking Reagent
  50 mM Na-phosphate, pH 7.0
  0.1% (w/v) N-lauroylsarcosine

See Appendix C for preparation of the "High SDS buffer"

Prehybridization, hybridization and blocking (during subsequent detection) can be performed in hybridization bags, dishes or heat-stable plastic bags.

Figure 11: You may use sealable containers, glass baking dishes (covered with plastic wrap or foil), or heat-sealable plastic bags.

Hybridization and washing conditions

Hybridization and washing conditions for DIG-labeled probes do not differ from those of radiolabeled probes. As in the case of radiolabeled nucleic acid probes, the optimal hybridization and washing conditions for each DIG-labeled probe must be determined experimentally. In this User's Guide, we provide recommendations for hybridization and washing conditions. Use the conditions given with each application as a starting point. It may then be necessary to optimize conditions to obtain maximum sensitivity with your probe.

Storage and reuse of hybridization solutions

One of the major advantages of the DIG system is the stability of the labeled probe. After hybridization with the blotted target, the hybridization solution still contains large amounts of unannealed DIG-labeled probe. To save the hybridization solution for future use, simply pour the solution into a capped plastic tube, seal, and store at –20°C. The probe is stable for at least 1 year when stored at –20°C. For reuse, thaw and denature by heating to +95°C for 10 min. If the hybridization solution contains formamide, denature at +68°C for 10 min.

Stripping and reprobing

With the DIG system, membranes can be easily stripped and reprobed. To do this, refer to the procedures on page 59.

What to do next

Proceed to the appropriate hybridization technique in this User's Guide.

Colony and Plaque Hybridizations

The DIG System provides a sensitive and rapid method for detecting positive colonies or plaques in a heterogeneous background. Colony and plaque hybridization have been developed for rapid screening of bacterial and phage recombinant genomic libraries for specific DNA sequences. The bacterial colonies or phage particles are transferred to a Nylon Membrane. Alkaline treatment serves to lyse the colonies or disassemble the phage particles. The denatured DNA is then immobilized on the membrane. A digoxigenin-labeled DNA, RNA or oligonucleotide probe is used for hybridization. Detection is carried out with a colorimetric or chemiluminescent immunoassay.

Products required
Refer to Appendix C for details on preparing these additionally required solutions.

Solutions	Description
Denaturation solution 1 (for plaque hybridization and Southern transfer)	0.5 N NaOH, 1.5 M NaCl.
Denaturation solution 2 (for colony hybridization)	0.5 N NaOH, 1.5 M NaCl, 0.1% SDS.
Neutralization solution 2 (for colony and plaque hybridization)	1.0 M Tris-HCl, pH 7.5; 1.5 M NaCl.
Standard prehybridization buffer	5 x SSC, 1.0% (w/v) Blocking Reagent for nucleic acid hybridization, 0.1% N-lauroylsarcosine, 0.02% sodium dodecyl sulfate (SDS) (recommended for color detection). When using RNA probes, add formamide to 50% and increase Blocking Reagent* to 2% (w/v) (recommended in general for chemiluminescence detection).
Standard hybridization buffer	DIG-labeled probe diluted in standard prehybridization buffer.
2 x wash solution	2 x SSC containing 0.1% SDS.
0.5 x wash solution	0.5 x SSC containing 0.1% SDS.

*Added from the Blocking Reagent stock solution (100 mM maleic acid, 150 mM NaCl, pH 7.5, containing 10% [w/v] Blocking Reagent for nucleic acid hybridization). See "Preparation of Additionally Required Solutions and Buffers" in Appendix C.

Any type of DIG-labeled DNA, RNA or oligonucleotide probe can be used for colony and plaque hybridization. To avoid nonspecific hybridization, it is necessary to use a probe which does not contain any sequences homologous to the vector. The optimal hybridization temperature and probe concentration must be determined empirically. We give the following as general guidelines:

Probe type	Probe concentration	Hybridization solution	Temperature for prehybridization and hybridization*
DNA	5–25 ng/ml	Standard buffer Standard buffer + 50% formamide	Hybridize overnight at +68°C. Hybridize overnight at +42°C.

Probe type	Probe concentration	Hybridization solution	Temperature for prehybridization and hybridization*
RNA	100 ng/ml	Standard buffer + 50% formamide	Hybridize overnight at +50°C.
Oligonucleotide		Standard buffer	Hybridize for 1–6 h; hybridization temperature varies considerably and can be approximated by considering probe length and G plus C content. Hybridization with a tailed oligonucleotide should be performed with 0.1 mg/ml Poly (A) and 5 µg/ml Poly d(A) both in the prehybridization and hybridization solution to prevent non-specific hybridization signals.
tailed	0.1–2 pmol/ml		
end labeled	1–10 pmol/ml		

*The conditions given here are stringent conditions applicable if probe and target have 100% homology and a GC content of about 50%.

Table 6: Optimal hybridization conditions for different probe types.

1. Plate phages and lawn cells (or bacteria for colony screening) at near confluence on a 10 cm LB agar plate. Allow the phages to grow overnight at +37°C. Bacterial colonies should be allowed to grow until they have reached a diameter of 1–2 mm. After growth, chill the plates for 1 h at +4°C.
2. Place a nylon membrane on the cold agar plate and leave for 1 min for primary filter, 3 min for duplicate filter. Label the filter asymmetrically with a needle to record the orientation of the filter on the plate. Replicas of phage libraries are made directly this way. The original plates should be stored at +4°C. Nonradioactive detection should not be applied to colonies grown on nylon membranes; this can lead to false positive signals. After the colony lift, the colonies can be recovered by placing the masterplate back in the +37°C incubator for a few hours.

Processing on the filter:

1. Place the phage lifts on dry blotting paper for 5–10 min. The phage particles bind to the filter as it drys.
2. Place 3 sheets of blotting paper (approximately the size of the membrane) side by side on plastic foil, and saturate with denaturation solution, neutralization solution or 2x SSC.
3. Place the phage-lifts phage-side-up for 5 min on a blotting paper saturated with denaturation solution. Colonies should be placed colony-side-up for 15 min on saturated blotting paper. Be careful, that no solution remains on top of the membrane.
4. Place the phage or colony lifts on the blotting paper with neutralization solution for 5 min, transfer to the 2x SSC-saturated blotting paper and leave for 15 min.
5. Fix the DNA on the wet membranes by UV-crosslinking or baking onto the nylon membranes for 15–30 min at +120°C.
6. Only for colony hybridization is the removal of cellular debris necessary to remove background on colony hybridizations. This is conveniently performed by incubating the filter in 3x SSC/0.1% SDS with shaking for 1–3 h at +68°C. Gently wipe the surfaces with a moistened towel.

If you expect background to be unusually high, treat the filters with proteinase K (20 µg/ml) for 1 h at +37°C. For later immunological detection it will be necessary to inactivate the proteinase K by incubation for 5 min in PMSF (40 µg/ml) at room temperature, followed by two short washes in 2x SSC. Keep in mind that PMSF